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Short communication

Cultivable bacteria associated with infective propagules of arbuscular mycorrhizal fungi. Implications for mycorrhizal activity

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ABSTRACT

This study aimed to isolate and characterize bacteria associated with surface-sterilized germinated propagules of arbuscular mycorrhizal (AM) fungi. It also aimed to evaluate their activity as mycorrhization helper bacteria (MHB) on the AM fungus *Rhizophagus intraradices*, which is commonly used in the formulation of bioinoculants. Most isolated bacteria did not significantly affect the viability and subsequent growth of mycelia. *Azospirillum* sp., *Rhizobium etli, Bacillus megaterium*,*Bacillus* sp., and *Paenibacillus rhizosphaerae* significantly enhanced pre-symbiotic variables (the re-growth/germination and the mycelia formed from AM propagules). *P. rhizosphaerae*, *Azospirillum* sp., and *R. etli* also increased extraradical mycelial length, mycorrhization percentages and the number of newly formed spores. The isolated MHB were characterized based on their starch-degrading ability, indole acetic acid production, phosphate solubilization, and inhibition of phytopathogenic fungal growth. Results suggest that some of the MHB studied, in association with viable AM propagules, could be potentially used as complex microbial inoculants for plant growth promotion.

1. Introduction

Most plant roots are colonized by arbuscular mycorrhizal (AM) fungi, obligate biotrophs that generally stimulate plant growth. After a symbiotic establishment with host roots, AM fungi produce a large number of infective propagules: spores, extraradical mycelium (ERM) and endophytic intraradical mycelium (IRM). These propagules are the source of inoculum for AM establishment in pure cultures, their viability is critical for successful cultivation. Fungal structures form the 'mycorrhizosphere', an additional habitat with characteristics that are different from those provided by roots. This habitat would selectively influence the presence of certain bacteria (Marschner and Timonen, 2005). The association of bacteria with AM propagules drastically influences the successful establishment of mycorrhization in both *ex vitro* and *in vitro* cultures.

Both culturable and non-culturable bacteria have been respectively isolated (Xavier and Germida, 2003) or detected by molecular methods (Roesti et al., 2005; Scheublin et al., 2010) in association with AM structures. The formation of bacterial biofilm-like structures on the surface of AM hyphae (Silvani et al., 2008; Lecomte et al., 2011) and the attachment of bacteria to AM spore walls (Cruz and Ishii, 2012) have been frequently observed. Mycorrhization 'helper' bacteria (MHB) include any strain capable of directly or indirectly promoting mycorrhizal symbiosis. MHB may increase propagule germination and hyphal growth, as well as stimulate spore production (Xavier and Germida, 2003). Many MHB are also plant growth-promoting rhizobacteria (PGPR) that enhance plant nutrient acquisition. Phosphate-solubilizing bacteria also show synergistic interactions with AM fungi (Fernández Bidondo et al., 2012). PGPR also produce auxins, which are able not only to stimulate differentiation and growth of plant tissues, but also to promote development of pre-symbiotic AM mycelium (Fernández Bidondo et al., 2011). Moreover, rhizospheric bacteria with antagonistic activity against fungal pathogens are able to promote AM formation (Budi et al., 1999).

The association of bacteria with AM fungi does not necessarily represent a benefit, as bacteria could take advantage of AM fungi in a trophic manner (Offre et al., 2006). Bacterial attachment to hyphae depends on the vitality of the fungal structures (Toljander

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et al., 2006). AM fungal inoculation stimulates chitinolytic activity in the rhizosphere (Abdel-Fattah and Mohamedin, 2000), and most bacteria attached to *Glomus* spp. spore walls are capable of degrading cellulose and chitin (Roesti et al., 2005). All these evidences indicate that AM fungal structures are a potential source of PGPR, but the compatibility among the isolated microbial strains should be tested, especially when complex biofertilizers (fungi and bacteria) are formulated, also including the host plant, to determine the effect of co-inoculations.

Sustainable agriculture maintains and enhances soil fertility and crop productivity through biological interactions and processes. Under sustainable crop management, AM fungi and plant growth-promoting MHB could be co-inoculated as biofertilizers, biocontrol agents and soil stabilizers. Since the development of an active rhizospheric community is essential for optimal plant productivity, knowledge of the interactions between functional microorganism groups is key to better understand plant-soil dynamics (Barea et al., 2005).

Although extensive studies have conducted on the benefits of the association between PGPR and AM fungi, very few studies have considered the diversity of PGPR naturally associated with the structures of these fungi. The impact of these bacteria on AM germination and infectivity dynamics, which are very significant events for AM *ex situ* germplasm conservation and for their effective application as biofertilizers, has also been scarcely discussed. Thus, the aims of this work were: i) to isolate and characterize rhizospheric and endophytic bacterial populations associated with infective AM hyphae and spores; ii) to analyze bacteria resistant to surface-sterilization processes, and their persistence upon the germination of fungal propagules (processes routinely involved in the isolation and conservation of AM fungal propagules); and iii) to assess their capacity as 'helper' bacteria in pre-symbiotic and symbiotic stages and their ability to promote plant growth, were also addressed to find possible candidates for complex biofertilizer formulations.

2. Materials and methods

2.1. AM fungi and bacterial strains

Bacteria were isolated from: (1) sporocarps of *Funneliformis mosseae* (strain G1), (2) spores of *Gigaspora margarita* (strain J5), and (3) IRM of *Rhizophagus/Glomus* spp. (BGIV collection of the School of Exact and Natural Sciences of the University of Buenos Aires (FCEyN, UBA)). One hundred sporocarps (G1) and one hundred spores (J5) were surface-decontaminated under axenic conditions (5%w/v Chloramine-T (Merck) solution for 30 min) and rinsed several times with sterile distilled water in line with Xavier and Germida (2003), sown on Petri plates with 0.35% w/v Gel-gro® (ICN Biochemicals, Aurora, OH, USA), and incubated for 10–15 days at 25 °C. Only the bacterial colonies growing in association with germinated spores/sporocarps after this time were transferred to tryptic soy agar (TSA) and re-streaked several times until pure cultures were obtained.

Bacteria associated with *Rhizophagus-Glomus* spp. strains were isolated from root segments (endophytic environment) from plants of several trap cultures (field soils) and processed as described by Silvani et al. (2008). Roots with visible intraradical AM fungal structures were selected under a stereomicroscope, surface-decontaminated under a laminar-flow bench with 3% v/v NaOCl for 3 min in Falcon® tubes, rinsed with sterile distilled water, cut into 3-mm pieces, and incubated on drops of 0.35% w/v Gel-gro® for 4 days at 25 °C. When re-growth of IRM from root fragments took place, bacteria associated with external hyphae were transferred to TSA medium as previously mentioned.

2.2. Characterization and identification of bacteria associated with AM fungi

Amylolytic, chitinolytic (Hankin and Anagnostakis, 1975), lipolytic (Sierra, 1957), proteolytic (Smibert and Krieg, 1994), cellulolytic, xylanolytic and pectolytic (Mikán Venegas and Castellanos Suárez, 2004) activities were detected using solid medium tests. A screening was performed to detect indole acetic acid (IAA) in the culture supernatants (Fuentes-Ramírez et al., 1993). The ability to solubilize inorganic phosphate was determined using NBRIP solid medium and sucrose or glucose as carbon source (Nautiyal, 1999) after 14 days of incubation. The *in vitro* antagonistic activity of isolated bacteria against *Macrophomina phaseolina* and *Fusarium solani* was screened. Diameters of fungal colonies in dual cultures were compared with those of the controls, and growth inhibition was calculated.

Bacteria were identified by 16S rDNA genes with the bacterial universal primers fD1 and rD1 (Weisburg et al., 1991). PCR-amplified 16S rDNA gene fragments (approximately 1.5 kb) were restricted with the endonuclease enzymes *Alu*I, *Hinf*I, *Dde*I, *Hha*I, *Hae*III, *Msp*I and *Rsa*I, and the lengths of the restriction fragments were determined by electrophoresis in 3% agarose gels. The restriction patterns (amplified rDNA restriction analysis: ARDRA) obtained from each isolate were compared. One strain of each ribotype was selected for bacterial identification. Sequencing was performed on an automated sequencer (ABI 3130xl Genetic Analyzer of Applied Biosystems) at the Sequencing and Genotyping Service of the FCEyN, UBA. Sequences were compiled using the BioEdit Sequence Alignment Editor 7.0 software, and compared to sequences from the GenBank database. Sequence similarity searches were performed using the BLAST server (http://www.ncbi.nlm.nih.gov).

2.3. Effect of bacteria on in vitro pre-symbiotic and symbiotic development

Viable and pure spores and mycelia of *Rhizophagus* intraradices strain GA5 (BGIV, http://www.bgiv.com.ar/strains/glomus-intraradices/ga5) were obtained from monoxenic cultures as described in Fernández Bidondo et al. (2011). Bacteria were grown in liquid BDN, centrifuged, and filter-sterilized (Millipore 0.2 μm pore size) to obtain diffusible substances (1). Pellets were re-suspended with 10 mM SO_4Mg to a final concentration of 10^9 cells ml^{-1} (2). Pre-symbiotic and symbiotic development of GA5 in response to 1 and 2 was evaluated.

2.3.1. Pre-symbiotic parameters

Groups of 30 spores or 10 colonized root fragments (1-cm long) previously removed from the GA5 monoxenic culture were transferred to Petri plates with 0.35% w/v Gel-Gro®. Each type of fungal inoculum was homogeneously mixed with 100 μl of (1) sterilized supernatants or (2) cell suspensions. Plates were incubated in the dark at 25 °C for ten days. All treatments were replicated in five Petri plates. The effect of bacterial strains on spore germination (%) and on re-growth of IRM from root fragments (%) was assessed under stereomicroscope (Nikon SMZ645). The ERM length (mm) was measured using the method proposed by Brundrett et al., 1994. Measurements were taken under a light binocular microscope (Nikon OPTIPHOT-T2) at 100x magnification.

2.3.2. Symbiotic parameters

A 1-cm^3 plug of a 3-month-old GA5 monoxenic culture, containing colonized roots (30% frequency and 50% intensity of colonization), approximately 250 spores and abundant ERM, was placed in proximity to fresh transformed carrot root explants in

Petri plates with 10 ml of MM. Then, 100 μl of (1) sterilized supernatants or (2) cell suspensions of some selected representative bacteria (strains SJ5R1, SJ5R2, SJ5R5, SJ5R7, TG1R2, TG1R4, BA2E, TaL1E, and TGX5E) was added. Plates were incubated in the dark at 25 °C for forty days. Each treatment was replicated in five Petri plates. The effect of bacterial strains on the ERM length of GA5 was measured using the grid intersect method (Marsh, 1971). The number of newly formed spores was assessed by counting 10 cells of 1 cm^3 for each replicate. The establishment of AM symbiosis was also checked: carrot roots were removed and stained with trypan blue (Phillips and Hayman, 1970). Intraradical colonization was quantified by examination of 50 randomly selected root pieces, and the frequency (%F) of mycorrhizal colonization was calculated as the percentage of root segments containing hyphae, arbuscules or vesicles. All measurements were taken under a Nikon light binocular microscope at 100x magnification.

2.4. Statistical analysis

Experiments were arranged in a completely randomized design with equal replications in each treatment. Effects of bacterial cell suspensions and diffusible substances (factors) on ERM length, number of newly formed spores, and frequency of mycorrhizal colonization of GA5 (response variables) were subjected to factorial ANOVA and comparisons among mean values were made using the least significant difference (LSD) test at $p < 0.05$. Statistical procedures were carried out with the software package STATISTICA 10.0 for Windows XP.

3. Results

3.1. Bacterial characterization

Results showed that 33.3% of J5 and 47% of G1 germinated propagules showed associated bacteria. Nine bacterial strains were recovered from the IRM of AM fungi, colonizing internal root tissues. According to the technique applied, unlike excessive surface disinfection (longer exposure time to NaOCl or antibiotic addition), the presence of endophytic bacteria did not affect the re-growth of IRM. The Supplementary material shows AM propagule germination (see Supplementary material SM Fig. S1 in the online version at DOI: 10.1016/j.apsoil.2016.04.013), origin, designation and identity of each bacterial isolate (see Supplementary material SM Table 1 in the online version at DOI: 10.1016/j.apsoil.2016.04.013).

Most bacteria were able to use starch as carbon source, half were able to degrade lipids, and only two were able to produce

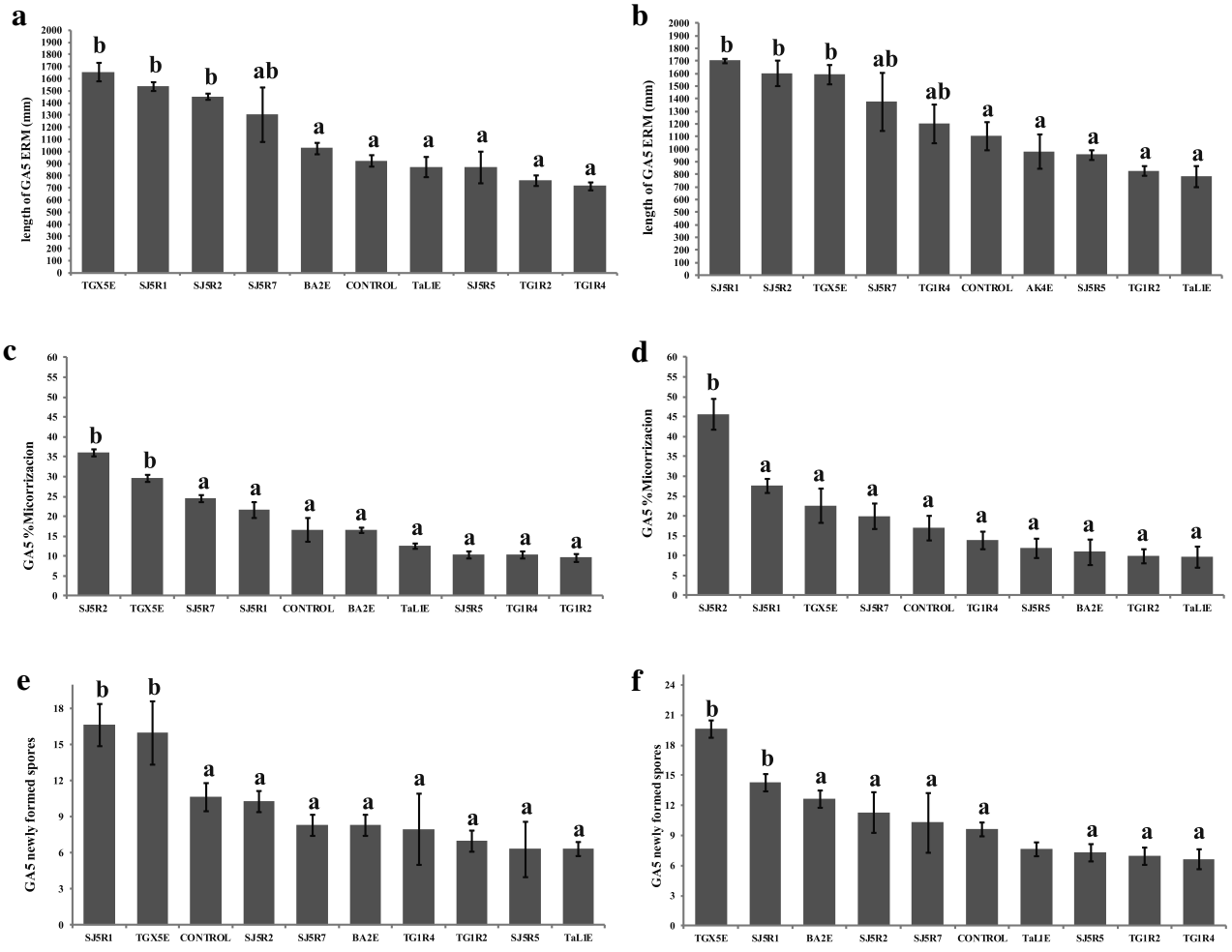

Fig. 1. Effect of bacterial cell suspensions on ERM length of *Rhizophagus intraradices* GA5 (a), frequency of mycorrhizal colonization (c), and number of newly formed spores (e), effect of bacterial diffusible substances on ERM length of *R. intraradices* GA5 (b), frequency of mycorrhizal colonization (d), and number of newly formed spores (f). Bacterial strain tested are presented on X-axis: SJ5R1 *Azospirillum* sp., SJ5R2 *Rhizobium etli*, SJ5R5 *Pseudomonas* sp., SJ5R7 *Bacillus megaterium*, TGX5E *Paenibacillus rhizosphaerae*, BA2E *Bacillus* sp., TaL1E *Pseudomonas* sp., TG1R2 *Paenibacillus favisporus*, TG1R4 *Paenibacillus* sp., Control: no bacterial treatment. Values are the means of five observations (±standard error). Bars with different letters are significantly different (LSD test, $p < 0.05$).

proteinase enzymes. All bacterial strains isolated from G1 sporocarps and only one isolated from J5 spores were able to degrade cellulose. Two endophytic isolates were able to degrade cellulolytic enzymes, and half were able to degrade pectins. *Paenibacillus* sp. strains were the only ones that showed xylanolytic activity. Only rhizospheric isolates exhibited chitinolytic activity: *Paenibacillus* sp. strains from G1 and *S. maltophilia* from J5. In addition, 64% of bacterial isolates were able to produce IAA and 48% of the isolates were able to solubilise inorganic phosphate with at least one carbon source. Six bacterial isolates were able to inhibit the *in vitro* growth of the pathogenic fungus *M. phaseolina*, while seven were able to inhibit *F. solani* growth (see Supplementary material SM Table 2 in the online version at DOI: 10.1016/j.apsoil.2016.04.013).

3.2. Mycorrhizal pre-symbiotic parameters

Cohnella sp. (BGA9E) and *P. favisporus* (TG1R1 and TG1R2) significantly decreased ($p < 0.001$) the percentage of spore germination to 50%, while *Azospirillum* sp. (SJ5R1) and *Bacillus* sp. (BA2E) significant increased this parameter with respect to the control. SJ5R1, BA2E, *R. etli* (SJ5R2), *Bacillus* megaterium (SJ5R7), and *Paenibacillus* rhizosphaerae (TGX5E) significantly increased ($p < 0.001$) the mycelial length produced by these GA5 germinated spores to about ±2400 mm. None of the bacterial strains had significant negative effects on the re-growth (%) or mycelial length of GA5 IRM. SJ5R1, SJ5R2 and *B. megaterium* (SJ5R7 and SJ5R6), and two endophytic strains of *P. rhizospherae* (TGX5E and BGA4E) significantly increased ($p < 0.001$) the germination of GA5 IRM. Strains SJ5R1, SJ5R2, SJ5R7 and TGX5E also significantly increased ($p < 0.001$) the length of GA5 IRM. The inoculation of bacterial exudates did not significantly affect GA5 spore germination or IRM re-growth. Bacterial diffusible substances did not significantly reduce the length of GA5 mycelia produced by germinated spores and root fragments compared to the control treatment. Exudates of SJ5R2 and SJ5R1 significantly increased ($p < 0.001$) the values of mycelium length from spores and root segments, respectively (see Supplementary material SM Table 3 in the online version at DOI: 10.1016/j.apsoil.2016.04.013).

3.3. Mycorrhizal symbiotic parameters

TGX5E, SJ5R1 and SJ5R2 significantly stimulated ($p < 0.001$) GA5 mycelial growth, more than 50% with bacterial cell suspensions (Fig. 1a), and up to about 65% with sterilized supernatants (Fig. 1b). Direct contact with TGX5E and SJ5R2 strains (Fig. 1c) and inoculation of SJ5R2 exudates (Fig. 1d) significantly increased ($p < 0.001$) the mycorrhization percentage values. Strains TGX5E and SJ5R1 (Fig. 1e) and their sterilized supernatants (Fig. 1f) also significantly increased ($p < 0.001$) the number of new spores per plate.

4. Discussion

A positive or neutral (not significant) ecological interaction between AM fungi and bacterial diversity hosted within viable and infective propagules was initially expected. The effect of bacteria on the viability of AM propagules and subsequent mycelial growth was mostly neutral and without significant alterations. It has been proposed that bacteria could interact with AM fungi by chemical mechanisms as well as by physically aggregating on spores and hyphal surfaces (Cruz and Ishii, 2011). This was confirmed herein, since MHB promoted AM fungal development by direct contact or by their exudates. Isolates that exhibited antagonist activity were also detected. It is likely that the method used could only isolate the most competitive and fast-growing taxa, which is a desired trait for the formulation of microbial biofertilizers.

The isolated bacteria belonged to species commonly found in the mycorrhizosphere and rhizosphere of mycorrhizal plants: *B. megaterium* and other *Bacillus* spp. (Lecomte et al., 2011), *P. rhizospherae* (Cruz and Ishii, 2011), and *Pseudomonas* spp. (Lioussanne et al., 2010). No phytopathogenic or human pathogenic bacteria were isolated, with the exception of *S. maltophilia*. This rhizospheric bacterium has been previously found in association with the ERM of *R. intraradices* (Mansfeld-Giese et al., 2002).

The bacterial population was characterized for its starch-degrading ability, the most important and abundant food reserve and energy source in plants. Many bacterial strains were also able to degrade lipids, stored in large quantities within the structures of AM fungi, demonstrating a trophic relation with fungal propagules. None of the strains characterized as MHB showed lipolytic activity. Extracellular enzymes released by bacteria could degrade plant cell walls, helping the fungus to penetrate into the root tissues. Bacterial biofilms associated with rhizospheric and endophytic AM fungal structures could be involved in root penetration, symbiosis formation, nutrient acquirement and protection against pathogens (Lecomte et al., 2011).

Ability to produce IAA compounds and solubilize inorganic phosphate, along with the ability to inhibit the growth of some fungal phytopathogens, could be interesting traits of the MHB isolated. For instance, *Azospirillum* sp. (SJ5R1), *R. etli* (SJ5R2) and *B. megaterium* (SJ5R7) act as MHB, stimulating germination of propagules and pre-symbiotic mycelial growth, both directly and by exudates. These plant-growth promoting capacities were also noticed in bacteria with detrimental effects. Some degree of specificity with AM species was observed. Strains of *P. rhizosphaerae* isolated from *F. mosseae* significantly increased sporocarp germination but inhibited spore germination of *R. intraradices*, while strains isolated from IRM were promoters of *R. intraradices*. Interestingly, *P. rhizosphaerae* has been recently found to be a probable endobacterium in *G. margarita* spores and to significantly increase its hyphal growth (Cruz and Ishii, 2011). Within *Paenibacillus*, bacteria seem to be the ones that could be considered MHB, as already confirmed by other *in vitro* (Horii and Ishii, 2006) and *in vivo* studies (Fernández Bidondo et al., 2012).

These data about specificity of interactions between AM fungi and MHB confirm the relevance of appropriate selection of microorganisms in the development of complex bioinoculants. This selection also ensures a permanent promoting effect on the survival and development of AM fungi, as well as final positive interactions on plant hosts.

Microorganisms closely associated with AM structures that remain after surface sterilization and persist during spore germination and hyphal re-growth could play an important role when AM fungi are able to resume their *in vitro* growth.

It is crucial to consider this complex microbial interaction and its biodiversity to sustain modern agricultural systems with the use of AM fungi and associated bacteria as biofertilizers.

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